Vol. 105, No. 2 October, 1953

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

FERTILIZATION AND ARTIFICIAL ACTIVATION IN THE EGG OF THE SURF-CLAM, SPISULA SOLIDISSIMA[1]

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In fertilization the sperm generally performs two functions: activation and syngamy; these functions are experimentally separable. Under conditions where artificial parthenogenesis appears to involve the same initial sequence of developmental events that follow fertilization, it is believed that the stimulating agent somehow imitates the activating action of the sperm. Consequently, the term *parthenogenesis* (natural or artificial) which implies cleavage and later development, has been included under the wider term *activation*. (For reviews on activation and fertilization, see Loeb, 1913; Dalcq, 1928a; Tyler, 1941; Lillie, 1941; Runnström, 1949; Brachet, 1950, Chapter IV; and Heilbrunn, 1952, Chapter XL.) In recent years egg activation has been considered also as a specific case in the general phenomenon of excitation. It is generally true that activating agents for eggs also are stimulating agents for other kinds of cells. For this reason it is logical to extend the same concepts of excitation to egg cells that have been applied to stimulation and response in nerve, muscle, or protozoan cells. Furthermore, work done on various phases of egg activation complements that being done on other kinds of cells.

A study of excitation requires that a response be detectable soon after application of the stimulating agent. The surf-clam egg has a great advantage in this respect, for it contains a large germinal vesicle, the membrane of which breaks down readily upon stimulation. Thus the breakdown of the nuclear membrane can be taken as a criterion of response. The presence of polar bodies or of pronuclei at about 45 minutes after activation was taken as evidence that the cell was alive.

Previous studies on artificial parthenogenesis, especially those involving the eggs of echinoderms, often employed cleavage as a criterion of activation. However, in *Spisula* eggs and other eggs where fertilization occurs before the onset of maturation division, activation is not always followed by cleavage.

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The purpose of the present study has been to make a preliminary survey of fertilization and artificial activation in the surf-clam egg. Wherever possible, the approach has been from the standpoint of similarities and differences in the mechanisms involved in fertilization and artificial activation. Because *Spisula* eggs have been little used in previous studies of this kind, it was considered desirable to make observations on normal and parthenogenetic development, and also to repeat experiments performed by others on different eggs in order to establish a baseline for further work on the mechanism of activation.

Schechter (1941) described a method for obtaining gametes from surf-clams, and also the structural changes which occur in aging eggs. His paper also contains some observations on normal early development. The clam used by Schechter was *Spisula solidissima* (Dillwyn), although it has commonly been called *Mactra.* Kostanecki (1908) described artificial parthenogenesis in the eggs of *Mactra stultorum* and *M. helvacea;* these eggs were obtained from animals collected at Naples, Italy. The eggs of the species of *Mactra* studied by Kostanecki appear to be quite different in many respects from those of *Spisula solidissima.*

I wish to express my gratitude to Dr. L. V. Heilbrunn, under whose friendly guidance these studies were carried out, for helpful advice on experiments and assistance in the preparation of the manuscript. I wish also to thank Dr. H. G. Borei for his helpful criticisms of the manuscript. Thanks are also due to Dr. S. Inoué for the use of his polarization microscope and air-turbine centrifuge. This study was greatly aided through the kind cooperation of Mr. and Mrs. P. Breslin and Mr. F. Matthews of Belmar, New Jersey, in obtaining surf-clams and sea water.

Material and Methods

A. Material

Along the New Jersey coast, eggs of the surf-clam can readily be obtained at any time from early spring until late autumn. At Woods Hole, Massachusetts, the season is shorter, beginning late in May and extending into September. At Woods Hole there seems to be a period of one or two weeks in the middle of the season when gametes are scarce; this period often coincides with periods of excessive summer heat. The spawning of surf-clams can be induced during the winter months by warming them gradually and feeding them plankton over a period of about two weeks (Loosanoff and Davis, 1950).

For the experiments at the Zoological Laboratory, University of Pennsylvania, surf-clams were transported from a float in the channel of the Shark River Inlet in Belmar, New Jersey to Philadelphia, where they were maintained under aeration at about 14° C. in a constant temperature bath for one to three weeks. Four clams about four inches in length can be kept for a period of five to six days in one half gallon of sea water in a jar. It is particularly important to evacuate any air trapped in the gill chamber of the clams; otherwise death will result in a short time. This may be done by holding and gently squeezing each clam under water with its siphons facing upwards until all air bubbles have emerged. Natural sea water was employed in maintaining clams, and artificial sea water of the same salinity was used in all experiments with eggs in Philadelphia. In Woods Hole,

filtered sea water or artificial sea water could be used for the experiments; clams were obtained from Barnstable and kept in tanks of running sea water.

1. *Determination of sex*

Dr. L. Thomas, in work done at this laboratory, found that by inserting a medium-gauge hypodermic needle between the valves, near the hinge into the visceral mass, gametes could be drawn out for microscopic examination. This procedure permits separation of the sexes if desired, saves time and material, and could also be used for obtaining small amounts of gametes.

2. *Obtaining gametes*

Large numbers of gametes are obtained by breaking the shell near the umbo, cutting the adductor muscles and removing the visceral mass intact. The gills, mantle, and heart are trimmed away to expose the gonad, which then is excised. A large portion of the gonad lies deep within the visceral mass toward the foot. An excised ovary is cut up in a small volume of sea water and strained through a pad of cheesecloth into a large beaker containing filtered sea water. The eggs are allowed to settle to the bottom and the supernatant fluid is siphoned off. This washing process is repeated at least three times (Allen, 1951a). Thorough washing removes most of the blood, cellular debris from the ovary, and fertilizin. The latter substance has an adverse effect on fertilization in this species (Allen, 1951c). Excised testes are stored in the refrigerator until needed. During this storage period (up to six hours) a milky fluid containing sperm flows out of the gonad. This sperm suspension ("dry sperm") may be drawn readily into calibrated pipettes, permitting an accurate measurement of sperm concentrations. Precise measurements of sperm concentrations are required for some aspects of work involving fertilization.

3. *Structure of the unfertilized egg*

The unfertilized *Spisula* egg has a diameter of 56 microns, measuring from inside the vitelline membrane. The jelly layer, which is about two microns thick, is thinner than that of most marine eggs. It can usually be observed only after the addition of a dye (such as toluidine blue or Janus green B) or as an invisible barrier separating crowded eggs. The tough, easily visible vitelline membrane surrounds a thin, clear layer which is optically empty under bright field illumination, but negatively birefringent in the radial axis of the egg. Beneath the thin, clear layer is a differentiated cortex containing large granules which fail to move when forces of 200,000 $\times$ gravity are applied with a Beams-type, air-turbine centrifuge. The cytoplasm is moderately granular and about 11% of its volume is occupied by fat-containing granules (centrifuge experiments). Centrifugation stratifies these fat granules centripetally in a dense layer long before the heavy granules begin to collect at the opposite end of the cell. The properties of the cytoplasm are such that movement of the nucleus by centrifugal force produces a ring of negative strain birefringence in the region of the cytoplasm through which the nucleus has passed (Allen, 1952a). The germinal vesicle is about 31 microns in diameter and is somewhat eccentric. In life the nuclear membrane exhibits

weak negative radial birefringence; when fixed, it shows, distributed along its inner surface, the entire chromosome complement except that chromosome which is attached to the nucleolus. The chromosomes are minute tetrads. The nucleolus is double (amphinucleolus), consisting of a transparent nucleolus and an opaque nucleolinus (see Plate I, Fig. E.). These structures are approximately 11 and 3 microns in diameter, respectively. Similar double nucleoli have been seen in many other marine eggs in the oöcyte stage.

Freshly shed eggs are irregular in outline. However, by the time washing has been completed, they usually become spherical. Presumably this irregularity in shape is due to compression in the ovary.

4. *Fertilization and early development at 21° C.*

a. *Optimal conditions for fertilization.* As mentioned earlier (see also Allen, 1951a), it is important to wash Spisula eggs several times with filtered sea water before insemination. Failure to wash sufficiently (*i.e.,* to remove blood and fertilizin, etc.) will result in low percentages of fertilization and delayed or inhibited cleavage. Two other important considerations are egg and sperm densities. Stender dishes containing 10 ml. of sea water were placed in a constant temperature bath. One or two drops of washed concentrated eggs were placed in the dish, so that their number was approximately 200–300 per ml. Immediately, 0.05 ml. of a fresh 1 : 500 dilution of "dry sperm" in filtered sea water was added to each dish. The sperm density varies considerably and should be assayed in a counting chamber for accurate work. The final density should be corrected to about 10^5 cells/ml.

The time taken for penetration of the sperm into different eggs in a population can be inferred from the sigmoid curves obtained by plotting counts of germinal vesicle breakdown against time after insemination. Fifty per cent nuclear breakdown occurs at about 9½ minutes after insemination; the spread of the curve is about 2–2½ minutes. As the time lag between sperm penetration and nuclear breakdown is the same in every egg, this curve indicates that all of the eggs are probably fertilized within 2–2½ minutes after insemination, depending on the chance meetings of eggs and sperm in the suspension. Furthermore, increasing the sperm concentration increases the slope of the sigmoid curve, but high concentrations also tend to produce polyspermy. The slope of the curve often permits detection of lots of poor eggs or sperm.

b. *Normal development at 21° C.* Soon after fertilization some eggs exhibit a small indentation at one point on the surface. This is more pronounced in some lots of eggs than in others. At best it is seen in about a quarter of the eggs in a microscopic field, but as in ordinary microscopic observation only a portion of the egg surface can be observed at any time, it is likely that most or perhaps all of the eggs show this irregularity. Almost simultaneously, the vitelline membrane elevates gradually from the surface of the egg. This process differs from the membrane elevation of echinoderm eggs, for the cortical granules, which appear to be so important in the membrane elevation of the sea urchin egg, do not appear to participate in the elevation of the *Spisula* egg membrane. The cortical granules persist after fertilization and throughout early cleavages.

Six or seven minutes after insemination[3] the nucleus begins to change; the transparent nucleolus dissolves and releases its substances into the nucleoplasm. The nucleolinus comes to lie free in the nucleus, and remains in the vicinity of the forming maturation spindle even after the disappearance of the nuclear membrane. Observations on fixed material indicate a spindle-forming role for the nucleolinus (Allen, 1951b).

The first meiotic metaphase lasts from 13 to 26 minutes after insemination. During this time, the spindle migrates from the center to the periphery of the egg. The first polar body is extruded at 29 minutes and its position establishes the animal pole of the egg. At this time the animal pole flattens slightly, then rounds up again at 35 minutes. The second polar body appears 39 minutes after fertilization. There follows a second brief flattening before the male pronucleus becomes visible at 50 minutes. Within another minute the female pronucleus also appears, and the two nuclei swell almost simultaneously, to a diameter of 11–12 microns before they merge and break down, giving rise to a clear region in the center of the cell. At 69 minutes the eggs elongate parallel to the long axis of the cleavage spindle. Formation of the cleavage furrow begins at 71 minutes, and is complete by 74 minutes. The nucleus re-forms in the (smaller) AB cell at 77 minutes and in the (larger) CD cell at 82 minutes after insemination. At 89 minutes both nuclei disappear again almost simultaneously in preparation for the second division at 99 minutes. The second cleavage divides the A and B cells equally, but the C cell is smaller than the D cell. Further cell divisions are more rapid and somewhat difficult to observe in living material. Rotating blastulae are seen usually within four or five hours after insemination; swimming larvae within a day. Veliger larvae will live at least three weeks in artificial sea water without feeding. (For early stages of normal development, see Plates I and II.)

c. *Abnormalities.* Polyspermic eggs usually fail to cleave; sometimes, however, they divide into blastomeres of equal size, or develop into abnormal larvae. Presence of more than two pronuclei in fertilized eggs is an indication of polyspermy.

Occasionally the cytoplasm of fertilized eggs becomes dark in color after nuclear breakdown. The cause of this abnormality may be sea water impurities, unripe gametes or unhealthy clams. Lots of clams with eggs showing this dark cytolysis often exhibit high percentages of spontaneous germinal vesicle breakdown after about an hour.

Aging in *Spisula* eggs leads to two obvious changes: first, there often appear deep indentations on the surface of the eggs (Schechter, 1941); such eggs can usually be fertilized normally if not too old. Second, the nucleus loses some of its normal transparency. Although these changes are not significant with respect to development, they should be recognized as a factor possibly influencing the physiology of the egg (see Scott and Hayward, 1950).

An occasional lot of eggs shows clumping of the cytoplasmic granules. Such eggs were discarded although it was observed that they were often able to develop normally.

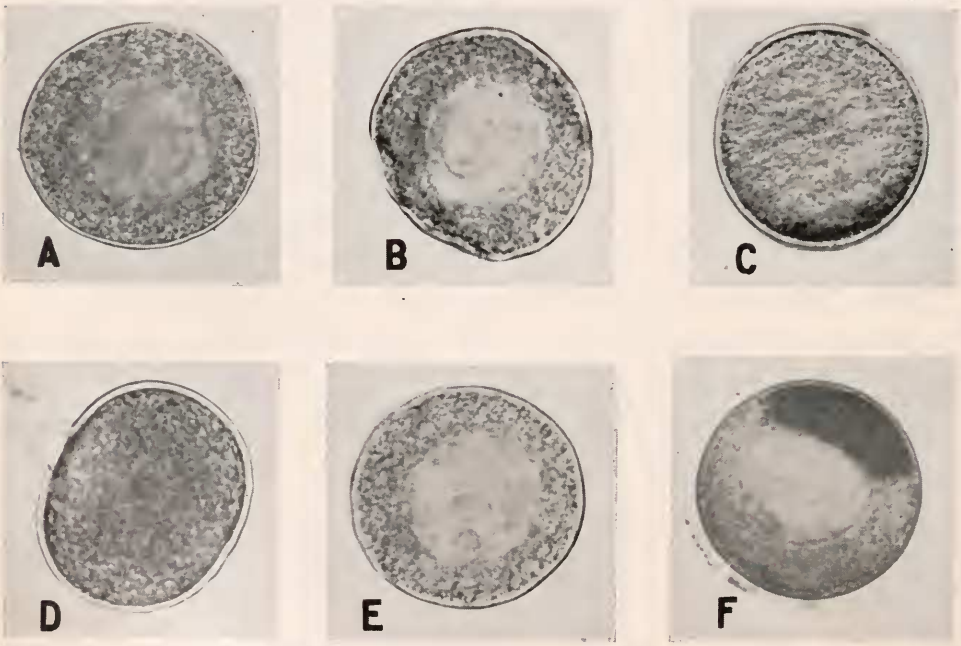
Under-ripe eggs often stick together in groups. This kind of agglutination

[3] All time figures given in this section refer to the time at which 50% of the eggs exhibit the particular phenomenon. This time is accurate to within a minute at constant temperature if egg batches from different females are compared under similar conditions. Under optimal conditions the spread of the points for eggs from a single female is about two minutes or less.

means that the jelly has not completely formed and is sticky. Such eggs are not suitable for experimental work, even though they may be fertilizable.

d. *Development of artificially activated eggs.* As far as could be determined, the early stages following artificial activation up to and including the breakdown of the nuclear membrane are identical with the early stages following fertilization. Furthermore, activated eggs usually form at least one polar body, often two; but with the methods tried so far, cleavage is rare. After one or two hours, activated eggs often exhibit from one to several nuclei, a condition highly suggestive of polyspermy. Such a multinucleate condition apparently comes about through a failure of the egg to form a second polar body; the second nucleus is retained in place of the male pronucleus. A similar situation was reported by Morris (1917) in the *Cumingia* egg, and by Kostanecki (1908) in *Mactra*. These nuclei sometimes undergo an abnormal mitotic reproduction without division of the cell; multipolar spindles are sometimes seen.

PLATE I. *Spisula solidissima* eggs



A. Unfertilized egg showing intact germinal vesicle.

B. Unfertilized egg shown 20 seconds after stimulation by isotonic sodium chloride in the presence of traces of calcium.

C. Egg 10 minutes after insemination showing the clear zone left after the dissolution of the germinal vesicle membrane.

D. Egg 30 minutes after insemination showing the first polar body; note the slightly elevated membrane (compare with B).

E. Unfertilized egg showing the double nucleolus in sharp focus.

F. A centrifuged egg showing the layer of light fat granules; note the sperm adhering to the surface of the jelly layer.

B. *Methods*

All experiments on artificial activation were carried out with widely dispersed eggs in order to eliminate crowding as a factor in excitation. A constant volume (10 ml.) of sea water or other medium was used throughout. Artificial activation experiments were carried out for the most part in petri dishes 5–6 cm. in diameter; water loss from evaporation was negligible. Experiments involving ether were carried out in stoppered vials. Since the threshold of stimulation depends on the temperature, this factor had to be controlled. For ordinary observations, room temperature (around 21 degrees) was sufficiently constant to eliminate errors from this source. For more exacting experiments a constant temperature water bath set at 21° (± 0.05°) was used.

The source of ultraviolet irradiation used in activation experiments was a Hanovia mercury arc lamp (Model number 7420) at a target distance of 35 cm. delivering approximately 4500 microwatts per square centimeter.

All solutions used in artificial activation experiments were isotonic to sea water with the exception of the mixtures used in osmotic stimulation. The exact isotonic concentration of sucrose used in centrifugation experiments could not be determined because the eggs always became very irregular in this medium. Unless stated otherwise, the pH of all solutions used was adjusted to 8.0–8.2 with 0.5 N NaOH. Mixtures of solutions and sea water are referred to as percentage (V/V) of isotonic solution in sea water (*e.g.*, "5% KCl-SW" means 5 volumes isotonic KCl and 95 volumes of sea water). In the figures, these percentage expressions have been converted to millimols of cation per liter of solution, since the concentrations of the other ions remain essentially constant.

Isotonic artificial sea water solutions were made up according to the formulas presented below, which were recalculated and modified after Lyman and Fleming (1940). Artificial sea water solutions lacking various of the cations were also used. For unbuffered solutions, bicarbonate was omitted. All solutions were prepared from water distilled in an all-Pyrex apparatus containing potassium permanganate and phosphoric acid.

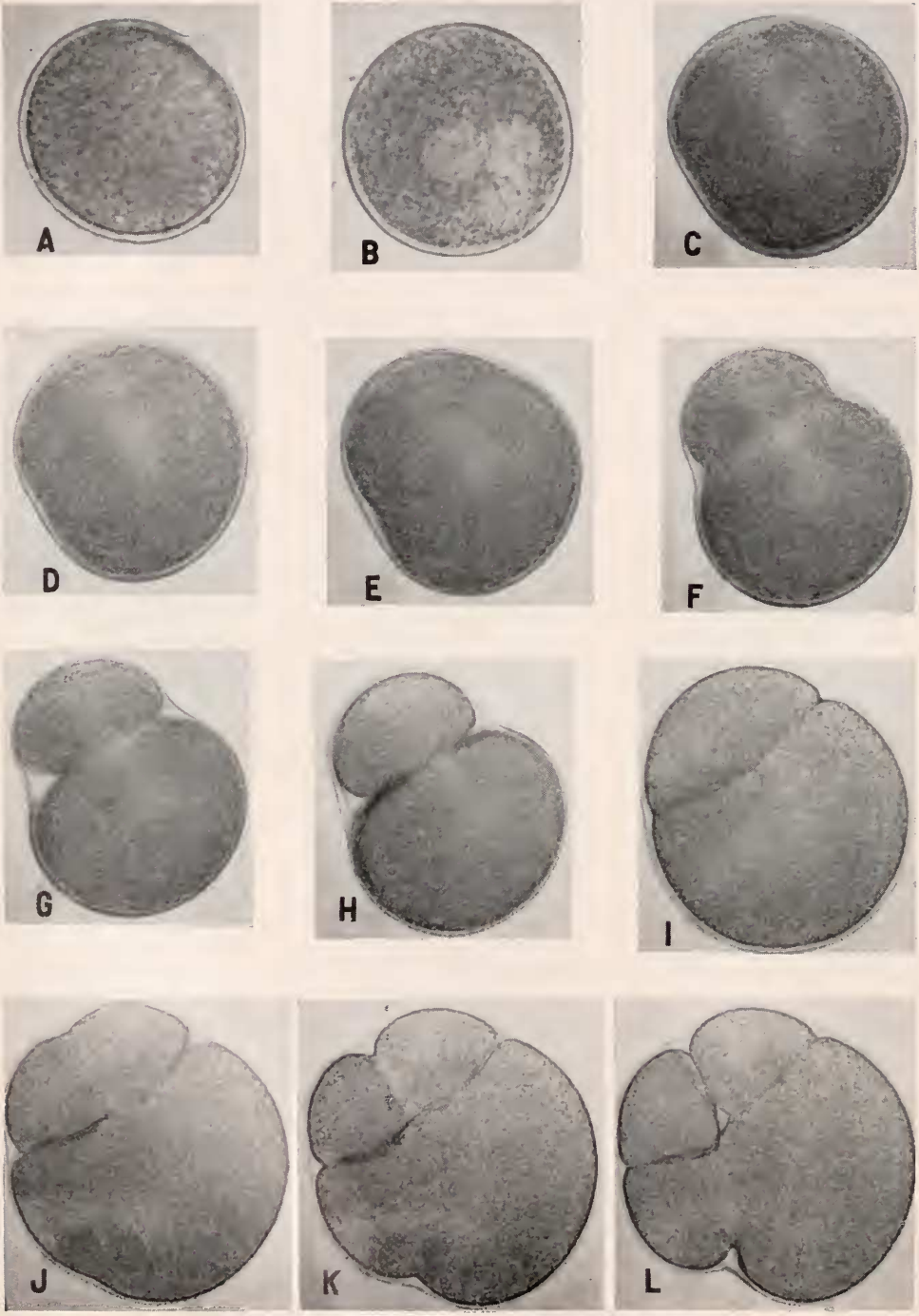
Formulas for approximately 4 liters of artificial sea water:

Substance	Sea water	Ca-Free	K-Free	Mg-Free
NaCl	93.90 grams	93.90	93.90	93.90
$MgCl_2$ ($6H_2O$)	42.35	42.35	42.35	—
Na_2SO_4	15.65	15.65	15.65	15.65
$CaCl_2$ ($2H_2O$)	6.10	—	6.10	6.10
KCl	2.65	2.65	—	2.65
$NaHCO_3$	0.76	0.76	0.76	0.76
Water	4076.0	3951.0	4003.0	3253.0

Isotonic solutions for Spisula eggs (from measurements of egg diameters):

Substance	Molar concentration
NaCl	0.50
KCl	0.52
$CaCl_2(2H_2O)$	0.35
$MgCl_2(6H_2O)$	0.34
Sucrose	0.73 (approximate)
Urea	0.96
Na citrate	0.35

PLATE II. *Spisula solidissima* eggs



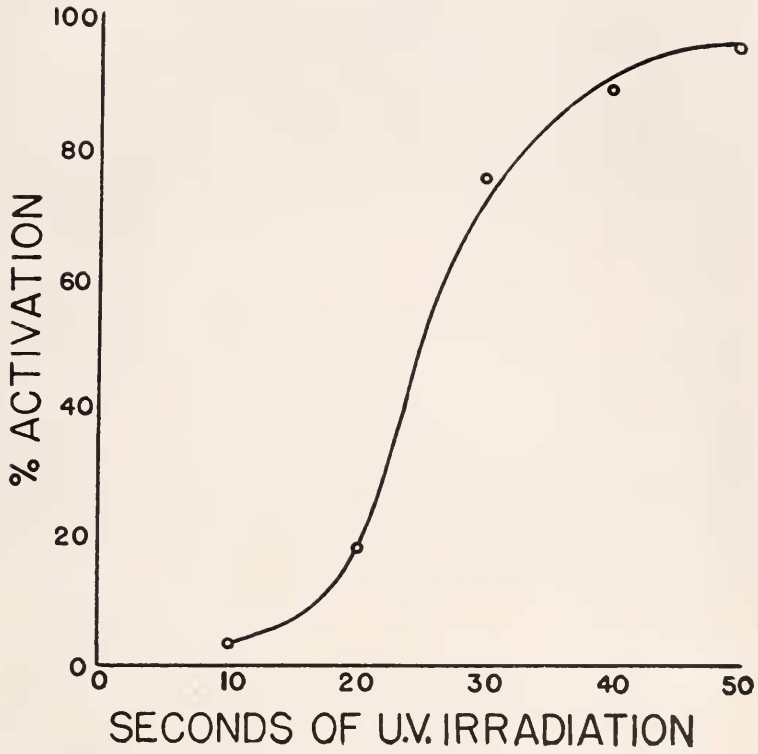


FIGURE 1. A typical population curve for a lot of eggs, obtained by plotting excitability against duration of exposure to the stimulating agent.

A sea water mixture containing only the chloride salts can be made by mixing isotonic chloride solutions in the following proportions: NaCl, 100 parts; KCl, 2.65; $CaCl_2$, 2.42; $MgCl_2$, 12.10.

RESULTS OF EXPERIMENTS

A. Artificial activation

Methods of activation were standardized to make possible a study of the influence on excitation of separate environmental factors and other agents. For this purpose curves were plotted for percentage of activation as a function of exposure time to the stimulating agent (usually ultraviolet light). The curve obtained in all cases was essentially one representing the distribution of excitabilities of individual eggs of a population. The shape and slope of the curve varied to some extent

A. Egg 43 minutes after insemination; note polar and clear region containing the spindle remnant.

B. The same egg 51 minutes after insemination; showing both pronuclei.

C–H. Photographs of a single egg at 21° C. taken at half-minute intervals from 71 minutes (C) to 73½ minutes (H).

I–L. Photographs taken of the same egg slightly compressed by a cover slide in order to show cleavage details. The time intervals between photographs was one-half minute, beginning at 93 minutes (I) and ending at 94½ minutes (L).

TABLE I

Artificial activation in Spisula eggs

Type of stimulation	Dose or concentration	Exposure time	No. of experiments	Maximum % activation	Reproducibility	Character of shape changes induced
U.V. light	4500 μWatts/cm.²	15 sec.–1 min.	116	100	excellent	Slight wrinkles at surface
Excess K^+	4–6% KCl-SW	4+ min.	69	100	good	Slight wrinkles at surface
K-Free SW	100%	1–4 min.	8	100	good	Slight wrinkles at surface
NH_3	10^{-4}–10^{-3} *M*	1–4 min.	12	100	good	Slight wrinkles at surface
Hypertonicity	100 ml. SW + 30–50 ml. 2.5 N NaCl	1–5 min.	15	100	fair	Deep indentations into the cytoplasm
Hypotonicity	10–20% SW	1–3 min.	78	100	good	Deep indentations on return to sea water
Heat	25–40° C.	10 sec.–15 min.	8	10	poor	Deep indentations (prolonged but reversible)
Cold	0–5° C.	10 sec.–15 min.	4	10	poor	Deep indentations
Urea	1–100% isotonic urea-SW	1–10 min.	10	5	poor	Sudden deep indentations
Protamine	50–200 mg. %-SW	30 min.–4 hrs.	6	100	fair	Shallow indentations

among different lots. Whenever difficulty was encountered in obtaining 95–100% activation by artificial agents, fertilization of the lot was usually found to be equally poor. Figure 1 shows an example of such a threshold curve for a lot of eggs.

A number of agents which have been employed in artificial parthenogenesis of various marine eggs will activate the eggs of *Spisula* (Table I). In most cases, it

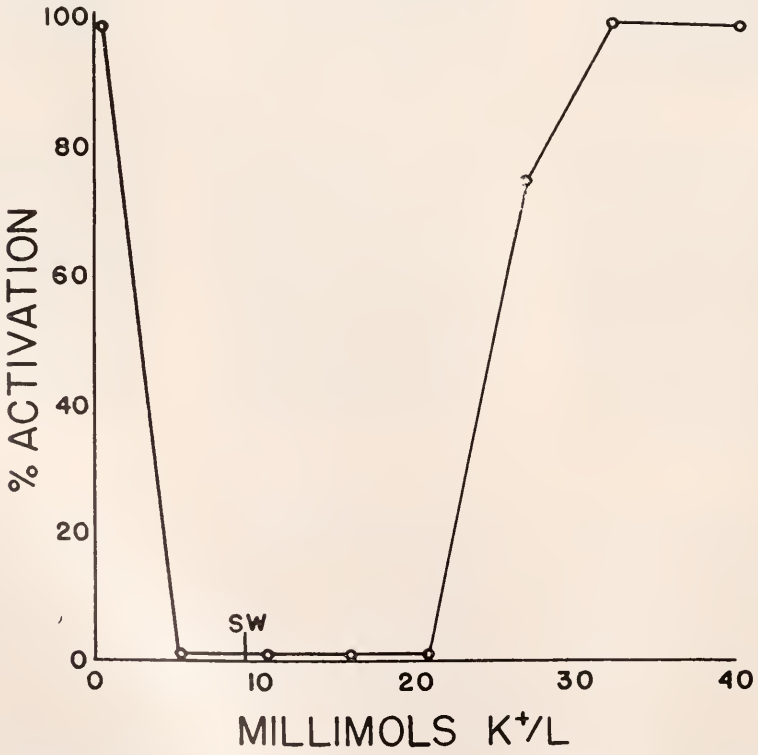


FIGURE 2. The activating effect of potassium ions above and below the concentration found in normal sea water.

was found possible to adjust the concentration of the activating agent so that only short exposure times were required for complete or nearly complete activation. Only in the case of excess potassium ions and of protamine (clupein) was it necessary to expose for longer than one to three minutes in order to obtain a result. Of the agents listed in Table I, only heat, cold and urea were distinctly unsatisfactory as activating agents for *Spisula* eggs. They are listed, however, for their interesting property of causing pronounced changes of shape in the eggs.

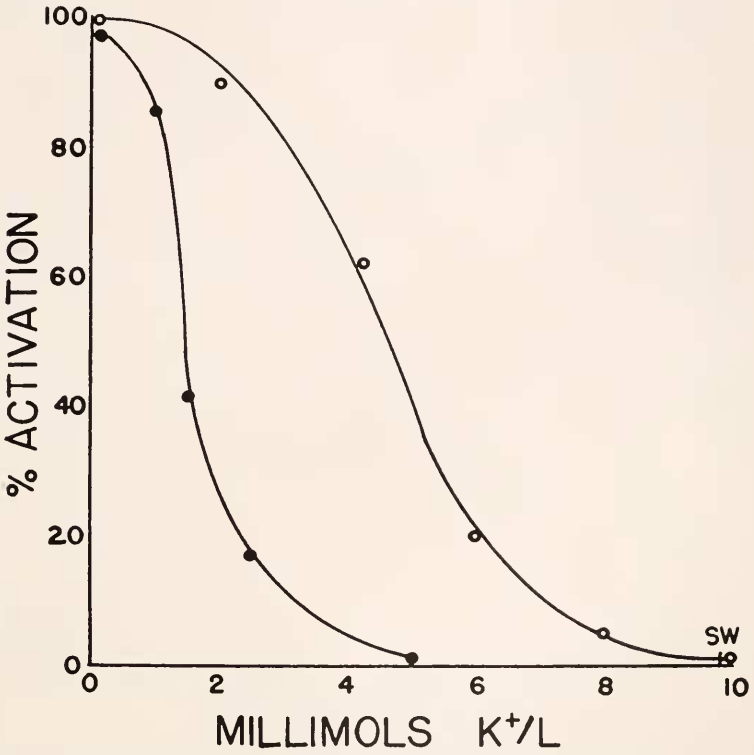


FIGURE 3. Curves showing (o) synergistic effect of low potassium concentrations in sea water and sub-threshold ultraviolet light stimulation, and (●) the activating effect of low potassium concentrations alone.

B. Changes in excitability following alteration of the medium

Experiments were carried out to determine the influence of changes in the environment on excitation, using an excitability curve (such as Fig. 1) as a guide to dosage. The concentration of each ion (except sodium) was varied while keeping tonicity and pH constant.

It was important to know how long eggs must remain in contact with a new medium before a change in excitability is manifested. The new environment causes the same degree of effect on the response whether the eggs are placed in it before, or up to four minutes after application of the activating agent. For this reason, in the experiments reported here, eggs were first stimulated by a dosage

of ultraviolet light (determined by a threshold curve) and then immediately transferred to separate dishes containing different amounts of the ion or other substance under consideration.

Even small increases or decreases in potassium concentration cause increased excitability. Both excess potassium and potassium-free sea water are excellent activating agents (Fig. 2). Figure 3 shows the effect of low potassium concentrations alone and in synergism with ultraviolet light.

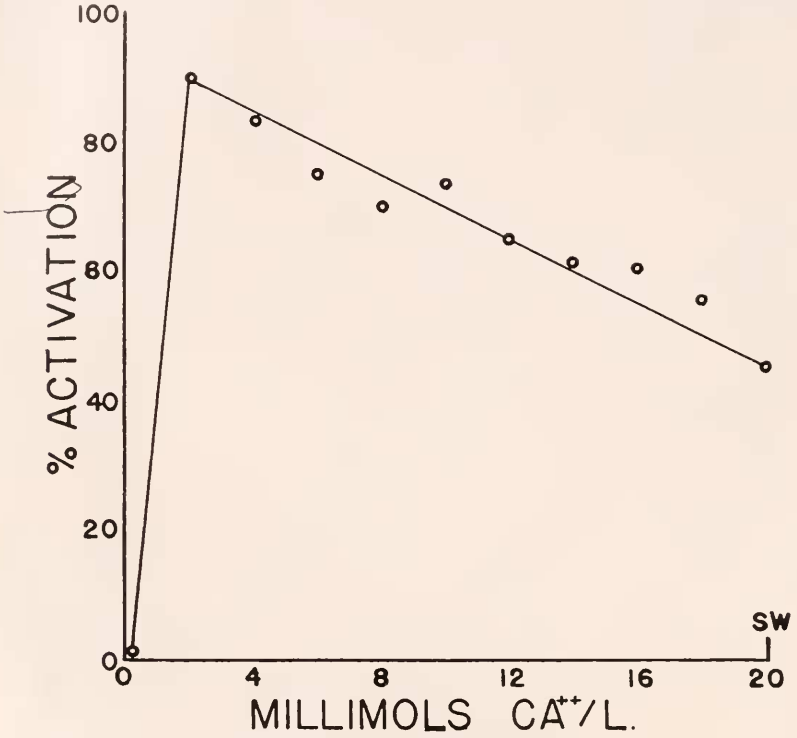


FIGURE 4. The effect of reduced calcium concentration on excitability (ultraviolet light stimulation). Dose of irradiation chosen to give 50% activation in normal sea water.

It was not possible to demonstrate an activating effect of any concentration of calcium. Calcium, however, apparently does enter and leave the egg, at least the cortical region, because even a slight alteration in calcium content of the medium causes a change in threshold to ultraviolet light stimulation (Fig. 4). Decreasing the calcium concentration results in a marked increase in excitability; a minimum amount of calcium is required for any excitation, however (about 5×10^{-4} M). Excess calcium ions reduce excitability. These effects are reversible.

If irradiation of the eggs by ultraviolet light in the absence of calcium is followed by transfer of the eggs into normal sea water, activation results. The percentage of activation is an inverse function of the time elapsed between the onset of irradiation and the transfer to the calcium-containing medium (Fig. 5). Other activating agents failed to give activation under these circumstances.

The effects produced by magnesium are quite similar to those of calcium as far as threshold is concerned. However, magnesium is apparently not necessary for activation. Figure 6 shows that excitability is high at low concentrations of magnesium and low when excess magnesium has been added.

Fertilization seems to be unaffected by changes in pH between 7 and 9 under conditions where ample sperm are present. At pH values below 7, the percentage of eggs activated by the sperm decreases rapidly. Below pH 5.0–5.2

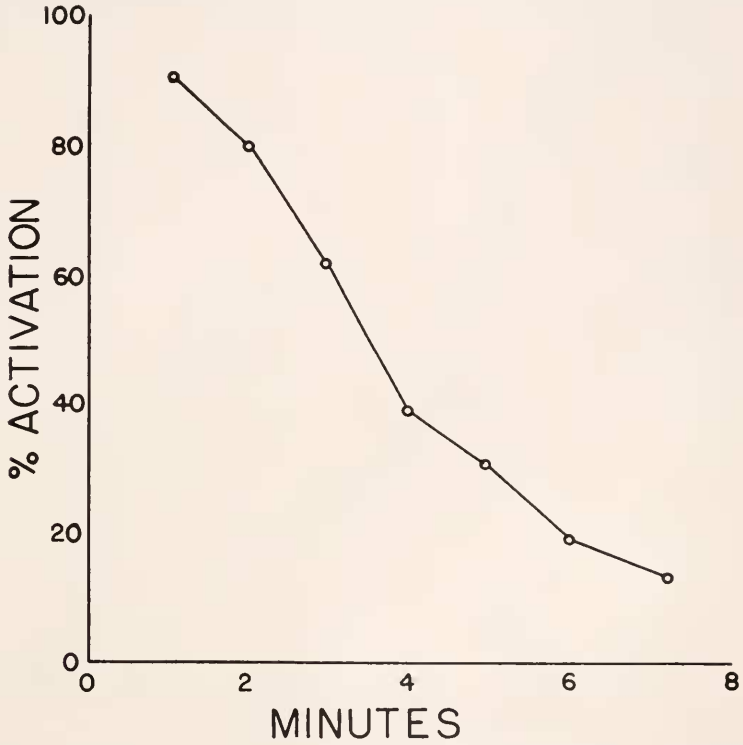


FIGURE 5. Activation obtained if eggs are irradiated in calcium-free sea water and subsequently transferred to normal sea water at the times indicated. Dose of irradiation chosen to give 100% activation in normal sea water.

there is usually no activation (Fig. 7). Using a strength of ultraviolet stimulation which at pH 8.0–8.2 activates 95–100% of the eggs, it is found that the pH at which total inhibition occurs is about the same as that at which fertilization is inhibited; see Figures 7 and 8. However, it appears that the shape of the pH-dependency curve differs for the three kinds of activation (sperm, ultraviolet light, and osmotic stimuli).

Since a rise in temperature accelerates the rate of many chemical reactions, it might be expected that heat would accelerate whatever reactions may be involved in excitation. In *Spisula* eggs, however, the opposite seems to be true; eggs stimulated at room temperature exhibit higher percentages of activation if trans-

ferred to sea water of lower temperature (Fig. 9). Similarly, eggs stimulated at low temperatures are inhibited if transferred to higher temperatures. On the other hand, previous exposure to heat (30–35° C.) or cold (0–5° C.) for a few minutes increases excitability when eggs are subsequently stimulated by ultraviolet light. It should be emphasized that heat and cold by themselves are not successful activating agents for *Spisula* eggs. These agents do, however, influence the

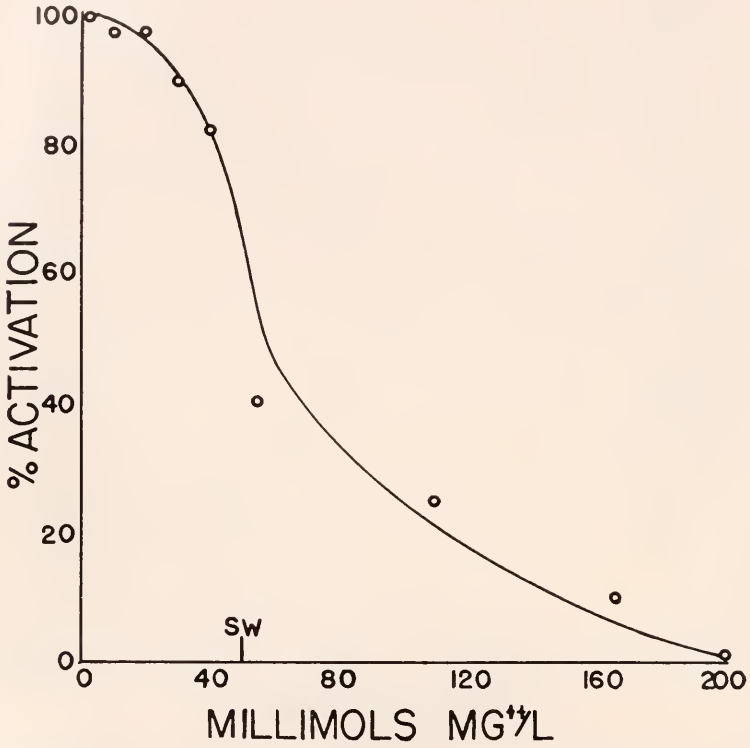


FIGURE 6. The influence of reduced and increased magnesium concentration on excitability. Dose of ultraviolet light chosen to give 50% activation in normal sea water.

response mechanism, as evidenced by their effects on the shape of the eggs and on excitability.

The anesthetics ethyl ether (Fig. 10) and ethyl urethane (Fig. 11) inhibit activation by sperm and decrease excitability for artificial activating agents. Chloroform causes extensive injury even at relatively low concentrations and therefore is not a good inhibitor for *Spisula* eggs. The egg jelly extracted with acid sea water and neutralized also exhibits a weak inhibitory influence on activation in sufficiently high concentrations.

Although normal fertilization causes only a slight and gradual membrane elevation, ultraviolet light in synergism with sea water of altered ionic constitution often causes a more pronounced elevation of the membranes. The monovalent cations are especially potent in this respect.

C. Duration of sensitivity to inhibitors

Fertilization in the presence of calcium could be reversed during the first four or five minutes of development by immersing the eggs in any of the following

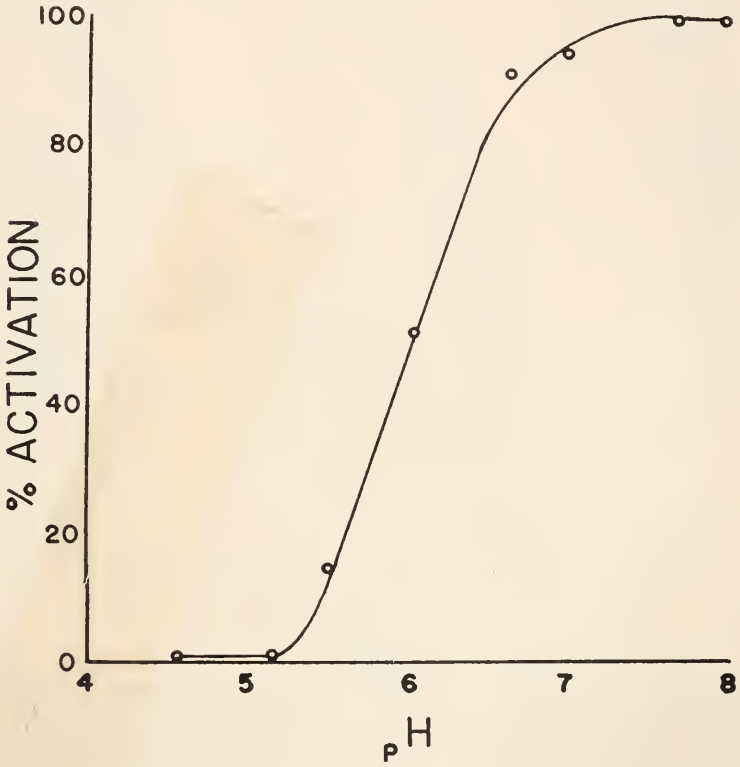


FIGURE 7. The influence of pH on fertilization (pH adjusted by adding HCl to bicarbonate-free sea water).

media: *a*, 10–20% isotonic sodium citrate [4] calcium-free sea water; *b*, acidified sea water (pH 5; made by adding 0.5 N HCl to unbuffered sea water), or *c*, 0.3–0.5% (by volume) ether in sea water. Developmental changes initiated during the first four or five minutes after fertilization could be reversed, because many of these eggs could be returned to normal sea water and activated a second time, this time by an artificial activating agent. These reactivated eggs often cleaved, in contrast

[4] Citrate is necessary to take care of the calcium carried over with the eggs; this concentration is well below the toxic level.

to the artificially activated ones which almost never cleave. Figure 12 defines the term "Stage I" which is the period during which eggs activated in normal sea water are sensitive to arrest by lack of calcium, lowered pH, or the presence of ether in their final medium. The time remaining until germinal vesicle breakdown is designated as "Stage II."

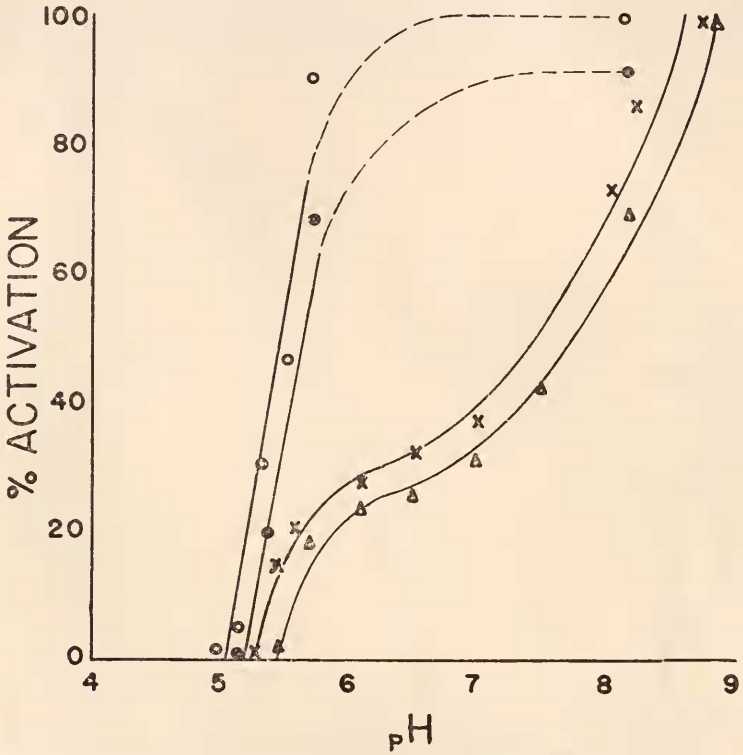


FIGURE 8. The influence of pH on artificial activation (pH adjusted by adding HCl to bicarbonate-free sea water). (o) represents 45 secs. ultraviolet irradiation; (●) represents 35 secs. irradiation; (×) represents hypotonicity, (△) represents hypertonicity. Dosages of stimulating agents arranged to give 90–100% activation at normal sea water pH.

D. Changes during Stage I

As mentioned earlier, slight changes of shape are often observed following sperm penetration or application of activating agents. Surface wrinkling or small indentations are often seen after application of some agents and deep indentations after others (*cf.* Tables I, II; Plate I). Artificial activating agents often evoke changes of shape more exaggerated than those resulting from fertilization. Eggs which are stimulated by ultraviolet light show marked wrinkling at the surface; sodium ions, isotonic urea (alone or in mixtures with sea water), heat and cold produce deep indentations which last from seconds to minutes. Such changes

in shape cannot be induced in the absence of calcium. Departure from spherical shape can be associated with changes in volume. In haematocrit experiments it was found that a decrease of at least 6–8% in volume follows stimulation by heat, urea, and sodium chloride if calcium is present. Decrease in volume means expulsion of water from the protoplasmic gel (syneresis). Such a gelation was detected by the centrifuge method after heat stimulation, which treatment also caused the

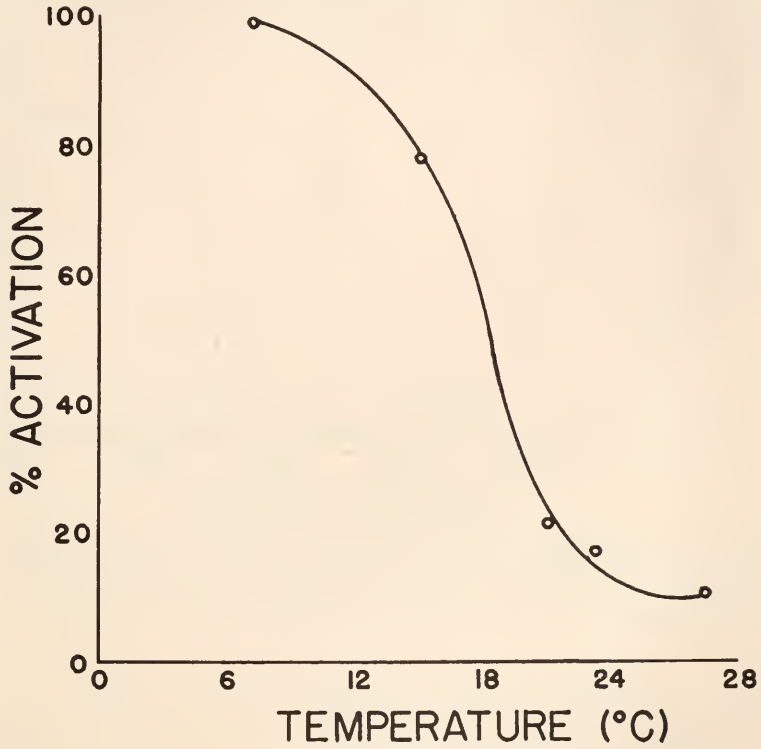


FIGURE 9. The effect of reduced temperature on excitability. The temperature of stimulation was 27° C. The dosage of ultraviolet light was chosen to give about 10% activation at this temperature. Aliquots of the stimulated eggs were transferred immediately to sea water at a lower temperature.

most prolonged indentations. The brevity of response to other agents made similar viscosity measurements by this method impossible. (Viscosity measurements are very difficult in unfertilized *Spisula* eggs because forces of the order of 200,000 times gravity are required to stratify the particulate matter in the cytoplasm.) There is a rapid increase in the rate of brownian movement at the end of Stage I; It is at about this time that the cell regains its spherical shape, probably through the uptake of water (imbibition).

The application of mixtures of two or three of the sea water cations in the proportions found in natural sea water shows some effects of ion antagonism on

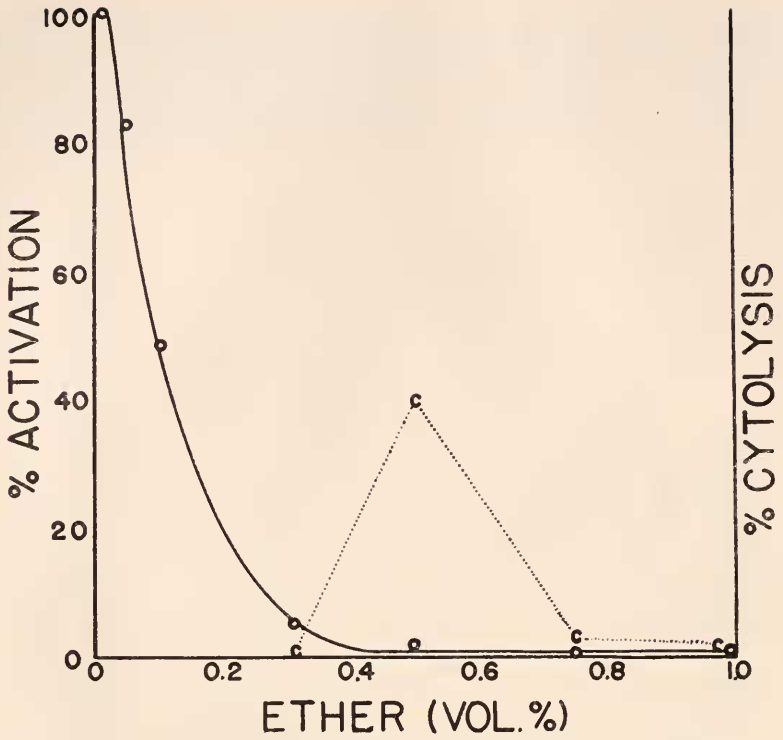


FIGURE 10. The inhibiting effect of ethyl ether. Eggs in sea water stimulated by dosage of ultraviolet light chosen to give 100% activation and subsequently transferred to sea water containing ether. The dotted line represents percentage of cytolysis (plotted on the same numerical ordinate).

TABLE II

Ion antagonism associated with changes of shape on stimulation*

Na^+	K^+	Ca^{++}	Mg^{++}	Wrinkling	Indentations
+	−	−	−	Marked	Few
+	+	−	−	Very slight	None
+	−	+	−	Marked	Marked
+	−	−	+	Slight	None
+	+	+	−	Few slight	None
+	+	−	+	None	None
+	−	+	+	Slight	None
+	+	+	+	None	None
−	+	+	−	None	None
−	+	+	+	Slight, prolonged	None
−	+	−	+	None	None
−	−	+	−	None	None

* Eggs were transferred with a minimum of sea water to mixtures of the isotonic cation chlorides. Thus in these mixtures, traces of all of the sea water cations were present. In the case of calcium, sufficient quantities of this ion were present to permit activation or changes of shape. The further addition of calcium intensified the shape changes.

changes of shape induced by sodium ions. Sodium ions in the presence of traces of calcium elicit sudden strong indentations. Table II shows that an excess of calcium intensifies the magnitude of the sodium response as long as the other cations are absent. Both potassium and magnesium alone weaken this response, and the two together abolish it. This is probably the reason that eggs do not normally show indentations when placed in sea water. It is true that freshly shed eggs are irregular in outline; this seems to be due to previous compression in the ovary. Hydrogen ions tend to prolong indentations caused by activating agents. Ether will not prevent indentations even though it inhibits germinal vesicle breakdown.

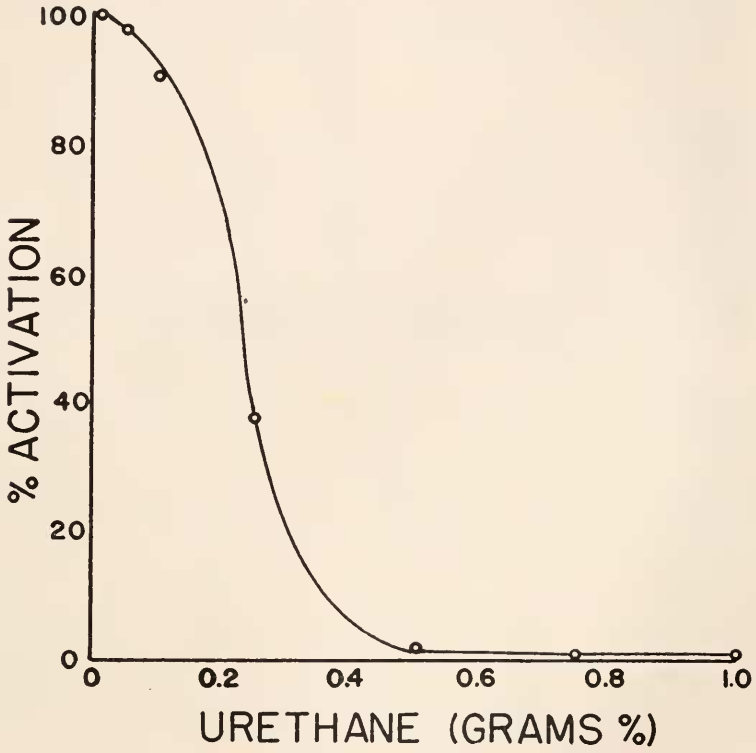


FIGURE 11. The inhibiting effect of ethyl urethane on stimulation by ultraviolet light. Dose of irradiation chosen to give 100% activation.

In fact, higher concentrations of ether can induce deep indentations. In living eggs, the removal of calcium by 10–20% sodium citrate in calcium-free sea water is the only way to prevent changes of shape following stimulation; this inhibition is reversible. Strong poisons may act similarly in preventing changes of shape, but their effects are not reversible. Thus iodoacetate (4×10^{-4} M but not 3×10^{-4} M) will inhibit changes of shape. Cyanide (10^{-3} M), or bromide (10^{-2} M) have no detectible inhibitory effect. Attempts at causing indentations with sodium ATP (up to 10^{-2} M) failed. A series of experiments was per-

formed in which eggs were transferred first to dilute sea water and then to various stimulating solutions of the same dilution (osmotic strength). Such a transfer of eggs from sea water to 50% sea water, and then to 50% isotonic urea resulted in surface indentations. Below this dilution, indentations were not detected. In another experiment indentations were observed following transfer from 35% sea water to 35% isotonic sodium chloride. In all of these experiments, and those summarized in Table II, the traces of calcium which accompanied the eggs when they were transferred from sea water to the test solutions were sufficient to permit changes of shape, and in some cases, nuclear breakdown.

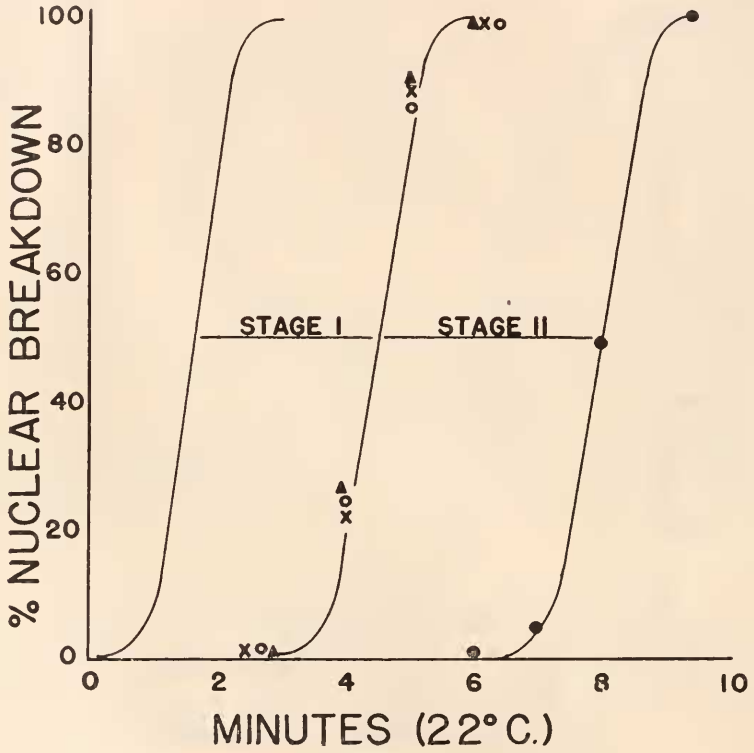


FIGURE 12. The duration of sensitivity to lack of calcium, acid sea water, or ether in eggs activated in normal sea water and subsequently transferred to these inhibitors. The right-hand curve represents the time at which dissolution of the germinal vesicle membranes is observed following fertilization. The left-hand curve is a hypothetical curve of the same slope, representing the probable penetration times of the sperm. The middle curve represents the time at which the fertilized eggs lose their sensitivity to inhibition by lack of calcium (o), acid sea water (×) and ether (▲). The time differences between the curves are called "Stage I" and "Stage II" as indicated in the figure.

During the course of Stage I there is measurable acid production in fertilized or artifically activated eggs. This is especially noticeable when eggs are suspended in unbuffered, bicarbonate-free sea water.

Discussion

A. Changes at the surface of the egg following activation

In the eggs of many marine invertebrates the penetration of the sperm causes striking changes at the cell surface. Although eggs of the sea urchin and other echinoderms exhibit highly elevated fertilization membranes, in general, the eggs of annelids and molluscs do not. There may be slight membrane elevation in these forms, however. Furthermore, in *Spisula* eggs, the cortical granules do not appear to be altered by fertilization or by artificial activation as they are in the sea urchin (Moser, 1939a, 1939b; Runnström, 1949), except perhaps under very severe conditions of artificial activation by ultraviolet light in synergism with an excess of one of the sea water cations. Under these conditions, widely elevated membranes are sometimes seen, and it is difficult to tell whether the cortical granules are changed or not.

The surface, especially the cortex of the egg, is important during the initial response of the egg to activating agents. Evidence for the importance of the cortex has been presented by Whitaker (1931), Runnström (1923), Monroy and Montalenti (1947) and by Wilson and Heilbrunn (1952). Smith and Clowes (1924), and Tyler and Schultz (1932) showed that a number of marine eggs could cleave normally at hydrogen ion concentrations greater than required to inhibit fertilization. This suggests that at least part of the fertilization reactions take place at the surface. Runnström and Kriszat (1952) believe that the cortex plays a "master role" in initiating a "chain reaction" at fertilization.

Some information concerning the role of the cortex can be inferred from the effects of various environmental changes on excitability. According to current opinion, the cortex is probably the only part of the cell into which ions from the environment can freely diffuse (*cf.* Mazia, 1940). Since minor changes in the ionic constitution of the sea water environment have an immediate and maximal effect on excitability, it seems logical to suppose that the part of the cell primarily involved in response reactions has come into a dynamic equilibrium with the new environment. It seems probable that these minor ionic changes must be acting primarily on the cortex to produce their effect so rapidly and drastically. Furthermore, minor changes in ionic environment which cause significant excitability changes have no detectible effect on cell division or later development. The fact that slight alterations in ionic balance tend to act synergistically with ultraviolet light to cause widely elevated membranes to appear indicates that these alterations do bring about cortical changes. That cell division proceeds in sea water modified in this way is evidence that the interior of the cell is not seriously affected. Thus the data on excitability changes under altered environmental conditions can be interpreted in terms of an effect on the cortex. These changes may well be related to the changes in cortical viscosity such as those described by Wilson and Heilbrunn (1952). However, other factors may be affected as well.

It is believed that, generally, the internal hydrogen ion concentration of a cell is maintained relatively constant over a wide range of external pH. Tyler and Schultz (1932) showed that fertilization was particularly sensitive to slightly acid sea water (pH 6.8–7.5). The excitability of *Spisula* eggs varied with external hydrogen ion concentration (Figs. 7 and 8). Accordingly, the curves of acid inhibition of activation could be interpreted as inactivation curves of some surface

enzymes, or processes occurring at the surface. Another possibility is that acid is affecting the dissociation of calcium from its protein binding, thereby disturbing the normal release of calcium following stimulation. Such a release of calcium could conceivably pass around the surface of the egg, as Moser (1939a, 1939b) believed, in a chain reaction similar to that postulated by Runnström and Kriszat (1952). However, the different curves of acid inhibition obtained with ultraviolet light on the one hand, and with osmotic stimulation on the other, might indicate somewhat different pathways for the response mechanism for these different stimulating agents, and for fertilization as well.

It has been shown (Fig. 2) that a lack of an excess of potassium in the normal environment can cause activation. Activation by potassium-free sea water is actually sodium activation. Although sodium and potassium ions both stimulate, they act antagonistically toward the cell. It may be primarily this antagonism which keeps the *Spisula* egg in the germinal vesicle stage until it is fertilized, but it is hard to imagine how the sperm or other stimuli could exert an effect by influencing this kind of a block, unless by a permeability change, for which there is at present no evidence in the *Spisula* egg. A similar situation of sodium-potassium antagonism appears to exist in the eggs of *Urechis* (Scheer and Scheer, 1947). Other factors, especially the divalent ions, pH and temperature doubtless also contribute to block activation. Wilson and Heilbrunn (1952) believe that either sodium or potassium can release calcium from its binding with protein in the cortex. Why these ions both stimulate (*cf.* results of Sawada, 1952, on *Mactra veneriformis*) and yet act antagonistically is not known. Sodium and potassium seem to act differently in their ability to initiate development (Allen, 1952b). The time required for nuclear breakdown is the same if eggs are activated by potassium (sea water with double potassium concentration) as if they are activated by sodium (potassium-free sea water). This indicates that developmental processes are initiated at the same time in both media. Eggs activated by sodium can be removed after a minute and developmental changes continue in sea water; eggs activated by potassium, however, must remain in the stimulating solution throughout Stage I (four to five minutes). If they are removed during this period and placed in sea water, the developmental processes already initiated will become reversed, as if they had been placed in an inhibitor.

Calcium plays an important role in the excitation of marine eggs and other cells. The necessity of calcium for fertilization and artificial activation is well known (see especially Loeb, 1915; Dalcq, 1928b; Pasteels, 1935). Of the parthenogenetic agents tried by Wison and Heilbrunn (1952), calcium was the only one which caused the cortex to stiffen. As a parthenogenetic agent, calcium is unusual in the length of exposure it requires to be effective. The results of this study (see Fig. 4) indicate that calcium enters the cortex and causes a decrease in excitability. Similarly, if calcium is withdrawn from the environment (and as a result, from the cell), excitability is increased. For many eggs, including *Spisula,* calcium is a poor activating agent. The parthenogenetic action of calcium in some eggs may be due to a leakage through the cortex; for, once within the cortex this calcium may imitate the effect of normal release of calcium by stimulating agents. The effects of calcium on excitability can be explained by postulating that agents which liquefy the cortex (*cf.* Wilson and Heilbrunn, 1952) act synergistically with one another and antagonistically with those agents, such as calcium,

which tend to stiffen the cortex. In this way, it would be possible to account for the action of the cations on excitability.

Magnesium acts as an anesthetic in *Spisula* eggs as in other cells (Wilbur, 1939; Sawada, 1952). Its effect on cortical viscosity has not been determined in marine eggs.

Ether is a good anesthetic for *Spisula* eggs and works best on activation when its concentration is below 0.3% (by volume). At slightly higher concentrations (about 0.5%) ether causes wide-spread cytolysis. At still higher concentrations this cytolysis is prevented, but the eggs will now cytolyze if returned to normal sea water. It is possible that ether causes cytolysis by some process which resembles stimulation, such as a release of calcium or an increase in permeability. The prevention of cytolysis at higher concentrations may be a direct inhibition of the enzymes responsible for disintegration of the cell.

The fact that activation in *Spisula* eggs is favored by reduced temperature (Fig. 9) does not support the theory of R. S. Lillie (1941 and earlier) for activation of starfish eggs. Much of the evidence gathered in this investigation is in opposition to Lillie's theory. Activation by some agents is so rapid in *Spisula* eggs that it is difficult to conceive of the formation of a complex activating substance such as Lillie postulated.

According to the colloidal theory of stimulation proposed by Heilbrunn and his students, all stimulating agents in general act on the same series of processes, namely, those which are involved in, or follow the release of calcium from protein binding in the cortex. Presumably the normal release of calcium would be brought about by a change in the binding capacity of the cortical proteins. An attempt was made to demonstrate that the binding capacity of these cortical proteins could be influenced by stimulating agents presented in the absence of calcium, with the result that subsequently added calcium would be able to initiate development. This was accomplished by ultraviolet stimulation (Fig. 5). Apparently the eggs recover from such ultraviolet effects, as evidenced by the decrease with time of their ability to respond to added calcium. Atttempts to duplicate these results with other activating agents failed.

The fact that egg excitability could be influenced after withdrawal of the stimulating agent, but only during the subsequent 4–5 minutes (Stage I), is apparently related to the fact that eggs are sensitive during this period to arrest by inhibitors (Fig. 12). Inhibition by acid at this stage suggests that a surface reaction is affected. Simultaneous susceptibility to lack of calcium suggests that this surface process requires calcium. It appears probable that the environmental changes and other agents which affect excitability do so by their influence on these surface processes occurring during Stage I The presence of such an inhibitor-sensitive period following fertilization was first shown by Tyler and Schultz (1932). Similar experiments were performed by Goldstein (1953) on the activation (spontaneous maturation) of the *Chaetopterus* egg. Goldstein found two stages which were inhibited by lack of calcium and by carbon dioxide, respectively; however, this maturation process is not inhibited by acid sea water (above pH 3.5), whereas fertilization is inhibited at a much higher pH (7.1) in this egg (Smith and Clowes, 1924).

Although from much indirect evidence it would be tempting to believe that all

stimulating agents work by initiating the same sort of response reactions, the following evidence seems to suggest that there may be variations in pathway to different agents: (1) the pH-dependency curves for activation by different agents seem to be dissimilar (Figs. 7, 8); (2) sodium and potassium, which act antagonistically in sea water, each cause activation when presented in excess or not antagonized. Furthermore, those ions seem to influence the activation processes differently. (3) Activation can be obtained if eggs are irradiated by ultraviolet light in the absence of calcium and subsequently transferred to normal sea water. These results could not be duplicated with other activating agents.

II. Changes in the interior of the egg following activation

Following fertilization, chemical changes in the interior cytoplasm and nucleus prepare the egg for its metabolic needs during development (for discussion, see Runnström, 1949; Brachet, 1950). Colloidal changes in the cell interior probably also play a role in the processes leading to cleavage and further development (see Runnström, 1949; Heilbrunn, 1952). The fact that calcium is required for changes of shape (see Table II) caused by stimulation indicates that this ion is involved in these colloidal changes. For reasons mentioned earlier, it was not possible to obtain direct information concerning cytoplasmic viscosity changes at activation. The resistance of *Spisula* eggs to stratification by centrifugal force appears to be due mostly to their particularly high viscosity. However, this resistance to stratification might also be due in part to a lack of any marked difference in specific gravity between the granules and the ground substance. This possibility was indicated by the fact that the fat-containing granules collect somewhat more rapidly at the centripetal pole than do the heavier granules at the centrifugal pole when the eggs are subjected to forces of about 200,000 × gravity for from one to three minutes. The fact that the cytoplasm is a gel is also indicated by the formation of a ring of negative strain birefringence around the nucleus when this structure is moved through the cell by centrifugal force.

Changes in shape (indentations) similar to those found in the present investigation were described in *Spisula* eggs by Schechter (1941) in connection with natural changes which occur on aging. He showed that these changes were brought about more rapidly when calcium was present in the medium. It is thus interesting that calcium is associated with the occurrence of indentations as a result of both aging and stimulation.

It has been pointed out above that the changes of shape observed are actually a visible expression of syneresis (loss of water) by the egg protoplasm. Some similar changes in shape were reported by Tyler (1932) in the egg of the echiuroid worm, *Urechis caupo*. This egg already possesses a polar indentation before fertilization. The indentation disappears, reappears, and disappears for a second time before the germinal vesicle breaks down. Tyler made measurements to show that volume changes are involved, and that as the first indentation disappears, there is a drop in viscosity. Possibly the second indentation in *Urechis* is similar to the indentation observed in the present study. Tyler explained the changes in shape and volume by assuming changes in internal osmotic pressure. Since viscosity changes seem to have been involved as well, syneresis and imbibition may have been the immediate factors involved in the shape and volume changes. Presum-

ably, the forces developed by a contracting gel could oppose those of osmotic pressure. This certainly is true in the *Spisula* egg, as mentioned earlier in the results, where deep indentations occur even when the stimulating media are diluted with distilled water. Contractions or wrinkling at the surface of eggs seem to be of rather general occurrence (for example, see Runnström, 1949).

The loss of water by the egg is followed after a few minutes by a return of water (imbibition) and a rounding up of the egg's contour. This entrance of water causes a sharp increase in the rate of brownian movement, indicating liquefaction. It is at about this time that the processes designated as Stage I come to an end.

The rapidity with which eggs undergo cytolysis with disintegration following excessive stimulation is an indication of the relationship of stimulation to the activation of enzymes in the egg (for discussion of this point, see Runnström, 1949). Goldstein (1953) who has studied the maturation process in the eggs of the annelid worm, *Chaetopterus,* suggests that the nuclear membrane is dissolved by a calcium-activated proteolytic enzyme. There is ample evidence for the presence of proteolytic enzymes in the egg cell after fertilization (Lundblad, 1944; Woodward, 1950; Gross, 1952). It has been observed (Allen, 1951b) that in several marine eggs, the breakdown of the germinal vesicle is preceded by dissolution of the nucleolar membrane. It is thus possible that if an enzyme is involved in nuclear and nucleolar breakdown, it may come from within the nucleus. Since such an enzyme would presumably be calcium-activated (because some calcium is necessary for nuclear breakdown) it could, as far as is now known about calcium-activated enzymes, be either proteolytic, as Goldstein suggests, or lipolytic.

Summary

1. The present study is a preliminary survey of fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima* (Dillwyn).

2. The structure of the egg, optimal conditions for fertilization, and normal early development of fertilized and artificially activated eggs are described.

3. The results of treatment by various parthenogenetic agents are discussed with particular reference to possible similarities and differences in their mode of action and pattern of response initiated. Among the agents discussed are: ultraviolet irradiation, potassium, sodium, ammonia, osmotic stimuli, heat, cold, urea, and protamine (clupein).

4. The influence of various changes in environmental conditions has been investigated and the results correlated with the function of the cortical region of the cytoplasm during the first four or five minutes immediately following stimulation. Monovalent cations, temperature shock, and stimulation in the cold all increase excitability. Divalent ions, stimulation at slightly elevated temperatures, lowered pH, or the addition of ether, urethane or egg jelly decrease excitability.

5. Sodium-potassium antagonism may be largely responsible for maintenance of the egg in the germinal vesicle stage prior to fertilization.

6. The changes which can be detected in the interior cytoplasm and in the nucleus following activation are discussed with particular reference to changes of shape and volume, and to nuclear breakdown.

7. It is concluded that shape changes are probably caused by an expulsion of water (syneresis) from the cytoplasmic gel when the egg is activated. This expul-

sion of water is probably due to an increased gelation caused by a release of calcium from the cortex by activating agents.

8. The response mechanism of the egg can be divided into two stages on the basis of the fact that for 4–5 minutes after activation it is susceptible to inhibition by acid sea water, by lack of calcium, or by dilute ether; but after this time inhibition by these agents is no longer possible.

9. Evidence concerning the direct cause of germinal vesicle breakdown is discussed, and this evidence suggests that a calcium-activated proteolytic or lipolytic enzyme is involved.

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